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Role of the Extracytoplasmic Function Protein Family Sigma Factor RpoE in Metal Resistance of *Escherichia coli*†

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RpoE of *Escherichia coli* is a sigma factor of the extracytoplasmic function protein family and is required for the expression of proteins involved in maintaining the integrity of periplasmic and outer membrane components. RpoE of *E. coli* was needed for full resistance to Zn(II), Cd(II), and Cu(II). Promoter gene fusion and quantitative real time reverse transcription (RT)-PCR (qRT-PCR) assays demonstrated that expression of RpoE was induced by metals. Global gene expression profiles upon metal treatment of a Δ*rpoE* mutant strain and its wild-type strain were analyzed with microarrays, and selected genes were confirmed by qRT-PCR. The absolute number of genes that were changed in their expression upon metal stress was similar in both strains, but the increase or decrease in transcript levels upon metal treatment was smaller in the Δ*rpoE* mutant strain than in the wild type. Genes showing increased expression in the Δ*rpoE* mutant strain encoded proteins that belong to general defense systems against protein-denaturing agents. Genes showing decreased expression were part of the RpoE modulon itself plus the *ompC* gene, encoding a major outer membrane protein. A Δ*ompC* deletion strain was as sensitive to Cu(II) and Cd(II) as the Δ*rpoE* mutant or a Δ*rpoE* Δ*ompC* double mutant strain. In the case of Zn(II), the double mutant was more sensitive than either single mutant. This indicates that increased expression of OmpC contributes to the RpoE modulon-mediated response to metals.

Sigma factors of the extracytoplasmic function family are part of the bacterial stress response regulon (18, 43). They react to stress signals outside the cytoplasmic membrane by transcriptional activation of genes encoding products involved in defense or repair processes (15, 47, 54). RpoE from *Escherichia coli*, present at six (± three) copies per cell (38), is a paradigm of these sigma factors (54). The products of the genes controlled by RpoE (RpoE modulon) are required for proper folding of outer membrane proteins and their turnover, phospholipid and lipopolysaccharide biosynthesis, signal transduction, expression of putative inner and outer membrane proteins (57), other envelope proteins such as DsbC, FkbA, Skp, and SurA, and recently identified Ecf proteins involved in extracytoplasmic function that were essential for growth of *E. coli* (15). RpoE might be even more important for survival of *E. coli* in the stationary phase than the "starvation" sigma factor RpoS (65). Because of the essential character of RpoE, Δ*rpoE* mutant cells seem to acquire a suppression that allows them to grow (19). However, the suppressor mutation has never been identified, although some essential genes under RpoE control are known (15).

RseA and RseB, encoded together with RpoE in the *rpoE-rseABC* operon, form a signal pathway that allows *E. coli* to respond to protein unfolding upon periplasmic or envelope stress, especially under heat shock conditions that lead to controlled proteolysis of RseA (3). Under nonstress conditions, the membrane-bound anti-sigma factor RseA sequesters RpoE through its N-terminal domain, thereby decreasing the cytoplasmic availability of RpoE for transcription initiation (14). The C-terminal domain of RseA interacts with the periplasmic protein RseB, which is present in about half as many copies per cell (about three) as RpoE. RseB binds to misfolded periplasmic proteins. Since RseB increases the affinity of RseA for RpoE 2.5- ± 0.5-fold, this binding event might titrate RseB away from RseA and lead to a release of RpoE from RseA (14).

Bacterial metal resistance is the result of an interplay of several metal efflux systems, which can mainly be assigned to the protein families RND (resistance, nodulation, cell division), CDF (cation diffusion facilitator), and P-type ATPases (46). There is accumulating evidence that this interplay is a two-step process that affects both cellular compartments, the cytoplasmic and the periplasmic space. In gram-negative bacteria, P-type ATPases and CDF proteins export their substrates across the cytoplasmic membrane into the periplasm. While P-type ATPases focus on the sulfur-loving elements Ag(I) and Cu(I) or Zn(II), Cd(II), and Pb(II), the CDF proteins transport the divalent metal cations of the first transition period from Mn(II) to Zn(II) plus Cd(II). In the second step, RND-driven transenvelope efflux complexes seem to export the cations from the periplasm across the outer membrane (46).

E. coli does not contain an RND-driven transenvelope efflux system for Zn(II) or Cd(II) export but detoxifies these two metal cations with the P-type ATPase ZntA and the CDF protein ZitB (23, 46, 56). On the other hand, copper homeostasis in this bacterium involves three components, CopA, CueO, and Cus. The P-type ATPase CopA transports Cu(I) from the cytoplasm to the periplasm (55, 56). The toxic effects of the presence of Cu(I) in the latter compartment, which probably originate from the oxidation of Cu(I) to Cu(II), are relieved by the multicopper oxidase CueO (24, 26, 58). The third copper homeostasis system, CusCBA/CusF, becomes important in a

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Δ*cueO* deletion mutant and under anaerobic conditions because CueO is probably dependent on oxygen for its function (51). CusCBA is an RND-driven copper transenvelope efflux system and CusF is a periplasmic auxiliary small chaperone for the efflux process (21, 22, 44). Copper stress is relieved in a Δ*cueO* deletion strain by the Cus transenvelope efflux system, which provides evidence for the importance of the RND-driven Cus system for periplasmic detoxification of copper ions (25). This marks the periplasm as an important cellular compartment for metal homeostasis in a way that has not been considered before. This study elucidates the contribution of the RpoE modulon to metal homeostasis in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study were derivatives of *E. coli* strain BW25113 (16) (see Table 8 in the supplemental material). For cultivations, either Tris-buffered mineral salts medium (40) containing 0.2% glycerol and 3 g/liter Casamino Acids (TGC) or Luria-Bertani broth (LB) (60) was used. Analytical-grade salts of metal chlorides were used to prepare 1 M stock solutions, which were sterilized by filtration. Solid Tris-buffered media contained 20 g agar/liter.

Genetic techniques. Standard molecular genetic techniques were used (45, 60). The Δ*rpoE* deletion strain was constructed with the gene deletion system described by Datsenko and Wanner (16). In the first step, a chloramphenicol resistance cassette was amplified from plasmid pKD3 with the primers FRT-rpoE-Ec-down and FRT-rpoE-Ec-up (Table 8 in the supplemental material). Lambda Red recombinase-mediated recombination was used to replace the *rpoE* gene in *E. coli* BW25113 with the *cat* gene. In a second step, the *cat* gene was deleted from the chromosome. Generalized P1 phage transduction was performed with the lysate from Δ*copA*::*cat*, Δ*cueO*::*cat*, and Δ*cusCFBA*::*cat* strains (25) and from Δ*zitB*::*cat* and Δ*zntA*::*cat* strains to yield multiple deletion strains.

For complementation of the Δ*rpoE* mutant strain, a 2,720-bp fragment containing the *rpoE* promoter, *rpoE*, *rseA*, and *rseB* was amplified from total DNA of *E. coli* K-12 with primers Ec-rpoE-Pst and rseB (Ec) pASK-Pst (Table 8 in the supplemental material), cloned into plasmid pAH125, and integrated into the chromosome of strain ECA101 as described (28). To construct promoter fusions with the *lacZ* gene, 500-bp promoter fragments of the genes *rpoE* (59) and *cueR*, *cueO*, and *copA* were amplified from total DNA of *E. coli* W3110 with primers Ec-rpoEp-Pst and Ec-rpoEp-Eco, cueRp-Pst and cueRp-Eco, cueOp-Pst and cueOp-Eco, and copAp-Pst and copAp-Eco, respectively (Table 8 in the supplemental material). These fragments were also cloned into *lacZ* reporter plasmid pAH125 and integrated into the chromosome of *E. coli* BW25113 as described (28).

Growth curves. Overnight cultures of the *E. coli* strains were diluted 1:400 into LB medium and incubated 2 h with shaking at 37°C. These cultures were diluted again 1:400 into fresh LB medium to give parallel cultures representing the test conditions and controls. These cultures were incubated at 37°C until stationary phase was reached. Growth was monitored as turbidity with a Klett photometer. To determine the plating efficiency, samples were diluted in LB, 0.1-ml volumes were plated onto LB plates, and the CFU were counted after 16 h of incubation at 37°C.

Dose-response curves describing the action of Cu^{2+}, Zn^{2+}, or Cd^{2+} on *E. coli* cells were also performed in LB medium. Overnight cultures of the *E. coli* strains were diluted 1:400 to inoculate parallel cultures with increasing metal concentrations. The cells were cultivated for 16 h with shaking at 37°C, and the turbidity was determined at 600 nm. The mean values of the data from at least three independent experiments were used to determine the 50% inhibitory concentration (IC_{50}) values, which are the concentrations required to diminish cell density by 50% under the conditions tested. The formula used was the simplified version $OD(c) = OD_0/(1 + \exp[(c - IC_{50})/b)]$ of a Hill-type equation as introduced by Pace and Scholtz (52) and described previously (5). $OD(c)$ is the turbidity at a given metal concentration, OD_0 is that at no added metal, b is the slope of the sigmoidal dose response curve, and c is the metal concentration.

Induction experiments. *E. coli* cells with *lacZ* reporter gene fusions were cultivated in TGC medium with shaking at 37°C. After 2 h, metal chloride was added at various final concentrations, and the cells were incubated with shaking for a further 2 h. Promoter activity was measured as β-galactosidase activity as described previously (41).

Microarrays of *E. coli*. *E. coli* cells, wild-type and Δ*rpoE* mutant strains, were treated for 10 min with 25 μM Cd(II), 100 μM Zn(II), or 250 μM Cu(II) or no metal as a control in TGC medium. The exposure time used was long enough to allow complete transcription of all *E. coli* operons up to 24 kb in size. However, it was short enough to see the initial response of the cells to the metal shock. The concentration was chosen to be high enough to see an effect at all but low enough to prevent artifacts resulting from a global metabolic breakdown rather than from a specific response of the cells to the metal treatment.

PAN *E. coli* K-12 V2 arrays (MWG Biotech, Ebersberg, Germany) were used, consisting of 4,239 gene-specific oligonucleotide spots and 48 spots with DNA from *Arabidopsis thaliana* that served as a negative control. Each set of conditions was performed four times, with two independent bacterial cultures and a dye swap according to the minimum information about a microarray experiment (9) protocol (see the supplemental material).

RNA isolation and preparation of labeled cDNA. Total RNA was isolated as described (27). After isolation, DNase treatment was performed followed by purification with RNeasy columns (Qiagen, Hilden, Germany). To exclude experimental artifacts resulting from DNA contamination, only RNA preparations that did not generate PCR fragments in a PCR with chromosomal primers without a previous reverse transcription reaction were used. RNA concentration was determined photometrically, and RNA quality was checked on formamide gels (60). In a reverse transcription reaction, 50 μg of total RNA was labeled in a 40-μl labeling reaction with 9 μg of hexamer primers, 50 μM either dCTP labeled with either Cy3 or Cy5 (Amersham, Freiburg, Germany), 0.5 mM each dATP, dGTP, and dTTP, 0.2 mM nonlabeled dCTP, 10 mM dithiothreitol, and 200 U of reverse transcriptase in reaction buffer (Superscript II, Invitrogen, Karlsruhe, Germany). Primers and RNA were heated to 70°C for 5 min and snap-cooled in ice. Reverse transcription proceeded for 10 min at room temperature, followed by 2 h at 42°C. To denature the remaining RNA 10 μl of 1 M NaOH was added and incubated for 10 min at 65°C, followed by addition of 10 μl of 1 M HCl. Nonincorporated fluorescent nucleotides were removed with a Qiaquick PCR purification kit (Qiagen, Hilden, Germany). The amount of cDNA and the integration of fluorescent dye were determined as published (8).

Hybridization of DNA microarrays and image analysis. Equal amounts of Cy3- and Cy5-labeled cDNA in 120 μl of hybridization buffer (salt-based, MWG Biotech, Ebersberg, Germany) were denatured for 3 min at 94°C and hybridized to the PAN *E. coli* K-12 V2 microarrays for 20 h at 42°C in a shaking waterbath. After hybridization, the arrays were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate and then in 1× SSC and 0.1× SSC. The slides were scanned with a laser scanner (Array Scanner 428, Affymetrix) seven times per channel with increasing photomultiplier settings to expand the dynamic range of measurements. The resulting images were analyzed with the ImaGene 4.2 software (BioDiscovery, Inc. El Segundo, Calif.). An average of the sensitivities was calculated by linear regression with MAVI Pro 2.6.0 software (MWG Biotech, Ebersberg, Germany).

Normalization. For normalization and obtaining ratios, two strategies were used, which both led to identical results. First we performed the algorithm with GeneSight version 3.0 (BioDiscovery). The signal intensities were background subtracted and normalized by the total array intensity. Scatter plot and histogram analysis were performed to obtain the ratio for each experiment. Second, our own algorithm was used, which provided more control over the steps of data processing. For a few genes, the results obtained by our method were compared to that of the commercial software. In all cases tested, the results of both procedures were similar.

In our algorithm, the number one minus the regression coefficient of the linear regression used to calculate the optimal photomultiplier sensitivity for image analysis was defined as the read error of a single data read. These single reads were normalized not against the overall brightness of the slide but against a probable number of 3,880 total gene-specific mRNAs per cell. This gave the expression level per experiment. The mean values of the expression levels of all four experiments of each data set were calculated. As the deviation, the standard deviation of this mean value was added to the sum of the four read errors to obtain the experimental error. When the results of the different conditions were compared, Q was defined as the ratio of the mean expression levels condition 2/condition 1. Additionally, Q' was used, with $Q' = Q$ if $Q > 1$, otherwise $Q' = 1/Q$. The significance was defined as (condition 2 − condition 1)/(deviation 1 + deviation 2). Results with significance > 1 or significance < -1 and $Q' \geq 2.0$ were further considered, and results with $1.5 \geq Q' > 2.0$ were kept as additional results if the significance met the same conditions.

Validation of microarray data by qRT-PCR. Microarray data were confirmed by performing quantitative real-time reverse transcription (RT)-PCR (qRT-PCR) on the iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, Calif.). The cDNA was produced with the same RNA used for microarray

TABLE 1. Resistance of *E. coli* mutant strains to copper[a]

Relevant genotype	Systems present	MIC (mM) of Cu^{2+}
Wild type	All	3.25
Δ*rpoE*	CopA, CueO, Cus	2.75
Δ*copA*	RpoE, CueO, Cus	2.25
Δ*cueO*::*cat*	RpoE, CopA, Cus	3.00
Δ*cusCFBA*	RpoE, CopA, CueO	3.25
Δ*rpoE* Δ*copA*	Cus, CueO	2.00
Δ*copA* Δ*cueO*::*cat*	RpoE, Cus	2.25
Δ*copA* Δ*cusCFBA*	RpoE, CueO	2.25
Δ*rpoE* Δ*cueO*::*cat*	Cus, CopA	2.50
Δ*rpoE* Δ*cusCFBA*	CopA, CueO	2.25
Δ*cusCFBA* Δ*cueO*::*cat*	RpoE, CopA	1.25
Δ*rpoE* Δ*copA* Δ*cueO*::*cat*	Cus	2.00
Δ*rpoE* Δ*copA* Δ*cusCFBA*	CueO	2.00
Δ*rpoE* Δ*cusCFBA* Δ*cueO*::*cat*	CopA	1.00
Δ*copA* Δ*cusCFBA* Δ*cueO*::*cat*	RpoE	1.25
Δ*rpoE* Δ*copA* Δ*cusCFBA* Δ*cueO*::*cat*	None	1.00

[a] MICs were determined on LB medium overnight at 37°C. The mean values of three independent experiments with identical results are shown.

analysis. For real-time PCR duplicate reaction with 1 μl of template cDNA, 5 pmol of primers, and the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) were used as well as a second independent approach including 1 μl of template cDNA, 5 pmol of primers, 0.5× SYBR Green (Fisher) and 1 U of *Taq* polymerase (Roche Diagnostics GmbH, Mannheim, Germany). Details for PCR protocols and primer sequences are available on request.

Fluorescence was measured at the end of each 72°C incubation and analyzed with iCycler iQ software (version 3.0). Melting curves analysis (60 to 95°C, 0.5°C increments) were performed to ensure PCR specificity. For quantification, standard curves of cDNA dilutions (1:10 to 1:10,000) were performed as duplicates. The crossing point for each reaction converted to log copy numbers was determined with the standard curve algorithm and arithmetic baseline adjustment. Expression ratios were obtained by dividing the copy numbers of two corresponding strains. An average of four copy numbers per cDNA as well as an average of two independent biological examples were calculated.

RESULTS AND DISCUSSION

RpoE of *E. coli* is required for full copper tolerance. The *rpoE* gene was deleted from the chromosome of *E. coli* strain BW25113 in a way that prevented polar effects on downstream genes. The copper resistance of a Δ*rpoE* mutant strain of *E. coli* was tested in solid (Table 1) and liquid (Fig. 1A) media. In general, and in agreement with the results of other groups, the MICs of copper were higher than those of other metals of the first transition period of the periodic system of the elements (22, 24, 25, 44, 48, 51, 55). To our current knowledge, *E. coli* does not contain cytoplasmic copper-containing proteins or a copper uptake system, but only CopA as a copper efflux system. Thus, due to the resulting slow import of copper combined with an efficient export and binding of copper to complexing components of this cellular compartment, the "free" copper in the cytoplasm reaches only zeptomolar concentrations (12). Therefore, usage of copper by *E. coli* and toxic actions of this element on *E. coli* may be exclusively periplasmic events without touching the cytoplasm, which could explain the comparable low toxicity of copper in growth experiments.

Deletion of *rpoE* led to a decrease in copper resistance. This suggested that RpoE-dependent genes were required for full cellular defense against excess copper. The differences in growth of a Δ*rpoE* mutant and its wild-type strain in dose-response curves (Fig. 1A) were the result of a longer lag phase rather than a lower growth rate (Fig. 2, Table 2) or growth yield (Fig. 2). The CFU count of Δ*rpoE* cells cultivated in the presence of 2 mM Cu(II) decreased after about 1 h for 2 h before it increased again (data not shown). The viability of wild-type cells, in contrast, was not influenced by 2 mM Cu(II). This demonstrated that the Δ*rpoE* mutant cells were killed by Cu(II) until a subpopulation of about 1% of the Δ*rpoE* mutant

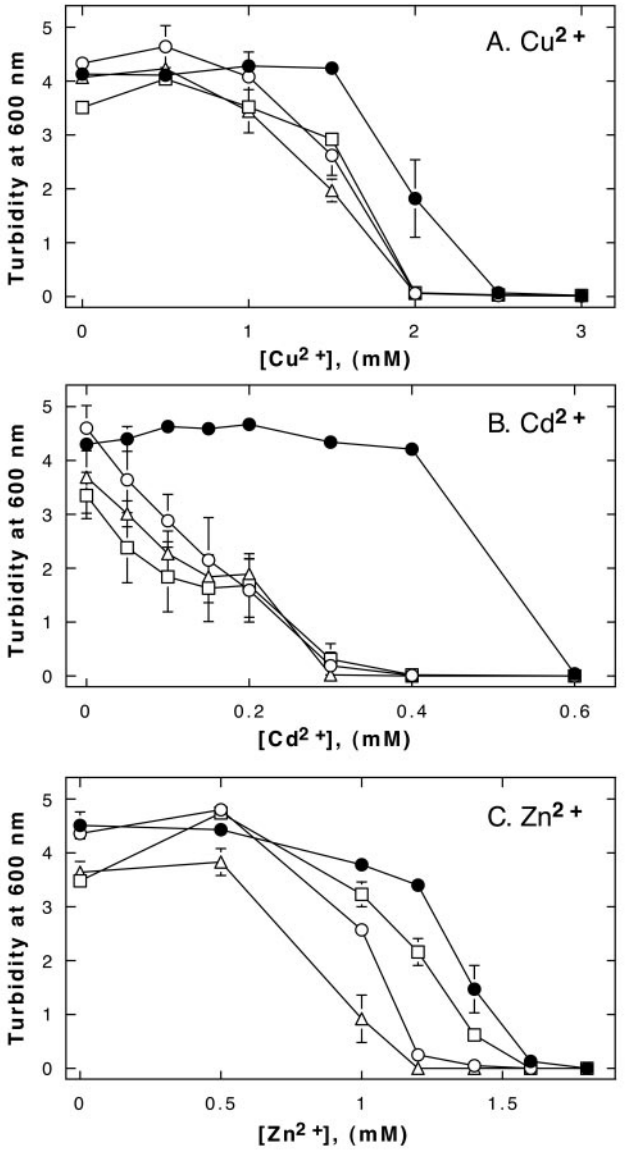

FIG. 1. Dose-response curves for growth of wild-type *E. coli* and its Δ*rpoE* derivative in the presence of metal cations. Parallel cultures of the Δ*rpoE* (open circles), Δ*ompC* (open squares), and Δ*rpoE* Δ*ompC* (open triangles) mutant strains and the wild-type (solid circles) in LB medium containing increasing concentrations of Cu(II) (A), Cd(II) (B), or Zn(II) (C) were incubated for 16 h at 37°C with shaking, and the turbidity at 600 nm was determined. The mean values of three experiments are shown with standard deviation bars.

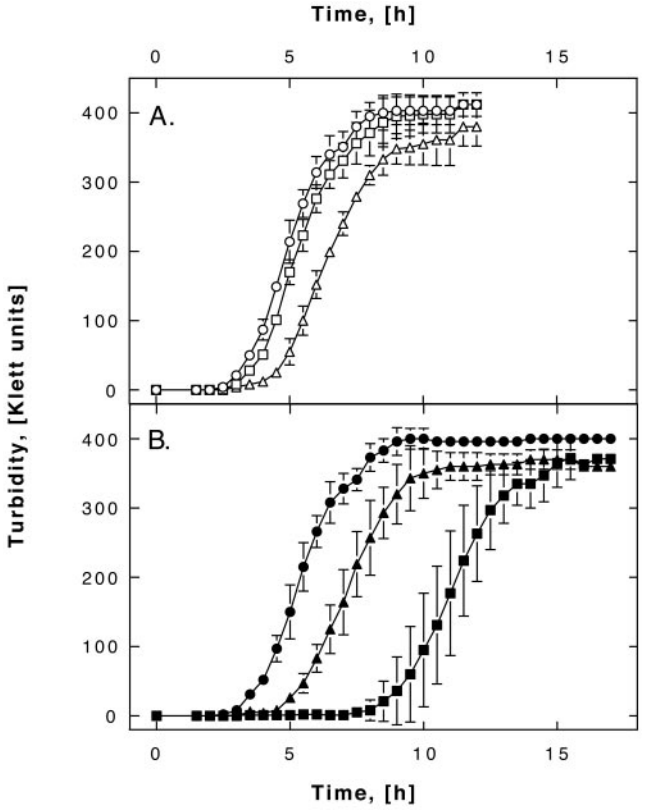

FIG. 2. Influence of copper on growth of a Δ*rpoE* strain of *E. coli*. Wild-type *E. coli* strain BW25113 (circles), its Δ*rpoE* deletion strain (squares), and a strain with the Δ*rpoE* deletion complemented in *trans* (triangles) were cultivated in LB without added copper (A, open symbols) or in the presence of 2 mM $CuCl_2$ (B, solid symbols), and the time-dependent increase in turbidity was determined. The mean values of three experiments are shown with standard deviation bars.

cells was able to mount a defense against the toxic metal. This subpopulation, however, was not that of a suppressor mutant, because cultivation of Δ*rpoE* cells in the presence of 2 mM Cu(II) followed by growth without added copper did not increase the copper resistance of these cells (data not shown).

Retarded growth of the Δ*rpoE* mutant strain in the presence of Cu(II) could be partially complemented by a single copy of the *rpoE-rseAB* operon inserted at the λ attachment site, λ_{att} (Fig. 2, Table 2). Partial complementation and retarded growth of the cells cultivated without added metals may be explained by the presence of the *rseAB* genes downstream of the deleted *rpoE* gene. This may lead to an imbalance between the RpoE sigma factor and its anti-sigma factor, resulting in an incomplete regulatory response to metal and other stresses.

RpoE is essential (15, 19), and an unknown suppression has to occur to allow growth of the Δ*rpoE* mutant strain. However, the stability of the copper-sensitive phenotype and partial restoration of copper resistance of Δ*rpoE* by complementation of *rpoE-rseAB* in *trans* indicated that the suppression allowed growth without added copper but did not restore copper resistance. This made it possible to analyze the influence of RpoE on metal resistance in *E. coli*.

The influence of a Δ*rpoE* deletion on copper resistance was weaker than that of a deletion of the copper-exporting P-type ATPase CopA (55) but stronger than that of an interruption of the periplasmic stress protein CueO (24) (Table 1). When the metal resistance of several different combinations of copper resistance gene deletions were analyzed, lack of all four genes (Δ*rpoE* Δ*copA* Δ*cusCFBA* Δ*cueO*::*cat*) led (as expected) to the lowest level of copper resistance (Table 1). Surprisingly, the presence of CopA alone (Δ*rpoE* Δ*cusCFBA* Δ*cueO*::*cat* mutant) did not increase copper resistance compared to that of the quadruple mutant. This indicates the importance of at least one copper homeostasis factor acting in the periplasm.

Regulation of copper resistance genes by RpoE. Gene fusions of *lacZ* with *cueO*, the gene for its regulator, *cueR*, and *copA* were constructed and inserted as single copies into the λ_{att} site of the bacterial chromosome of the Δ*rpoE* mutant and its wild-type strain. In both types of reporter strains, expression of all three proteins was induced by copper (Fig. 3). Thus, RpoE was not required for this process. However, the copper concentration needed for optimum induction of *cueR* or *copA* was lower in mutant cells, about 1.0 to 1.5 mM Cu(II) (Fig. 3A and C) than in wild-type cells, about 2.0 to 2.25 mM Cu(II). Cells able to survive at copper concentrations above a "threshold" concentration of about 1.5 mM Cu(II) needed a functional Cus system or CueO or RpoE (Table 1) to minimize copper toxicity in the periplasm. At copper concentrations above this threshold, a strong increase in copper-dependent expression of CueO was visible, but only in wild-type cells (Fig. 3B). Induction of CueO by copper followed a two-phase response that, below the threshold, was RpoE independent but, above it, was RpoE dependent (Fig. 3B). This inability to respond might be due to copper damage above the threshold concentration, which agrees with the bactericidal activity of 2 mM Cu(II) on Δ*rpoE* cells as observed in the growth experiments (Fig. 2).

RpoE is also required for full cadmium and zinc tolerance. When zinc or cadmium stress was analyzed on solid medium and in liquid culture (Fig. 1B and 1C and Tables 2 and 3), deletion of *rpoE* also showed a clear effect on resistance to either metal, indicating the necessity of RpoE-dependent factors for full tolerance against these two metals, as well. The effect of a Δ*rpoE* deletion was smaller than that of a deletion of the P-type ATPase ZntA but higher than that of a loss of the CDF protein ZitB (Table 3). However, in contrast to copper, the presence of ZntA alone led to a strong increase in zinc and cadmium resistance (Δ*rpoE* Δ*zitB* mutant), while the effect of the presence of RpoE or of ZitB alone (the other two double mutants) on metal resistance was similar. Nickel, cobalt, and

TABLE 2. Parameters of growth of a Δ*rpoE* mutant strain in the presence of metal cations in liquid culture[a]

Metal added and concn (mm)	Wild type		Δ*rpoE* mutant		Δ*rpoE* λ_{att}::*rpoE RseAB* mutant	
	t_d (min)	t_l (min)	t_d (min)	t_l (min)	t_d (min)	t_l (min)
None	38 ± 3	25 ± 11	35 ± 4	70 ± 28	41 ± 9	94 ± 69
$CuCl_2$ (2)	42 ± 7	27 ± 28	52 ± 6	301 ± 47	55 ± 11	58 ± 40
$ZnCl_2$ (0.7)	46 ± 7	36 ± 19	72 ± 8	288 ± 18	(47)	(487)
$CdCl_2$ (0.2)	50 ± 8	68 ± 19	55 ± 1	612 ± 12	(76)	(478)

[a] The mean values of three experiments performed in LB medium are shown with standard deviations except that single values are from are experiment. t_d, doubling time during the exponential phase of growth; t_l, time of lag phase.

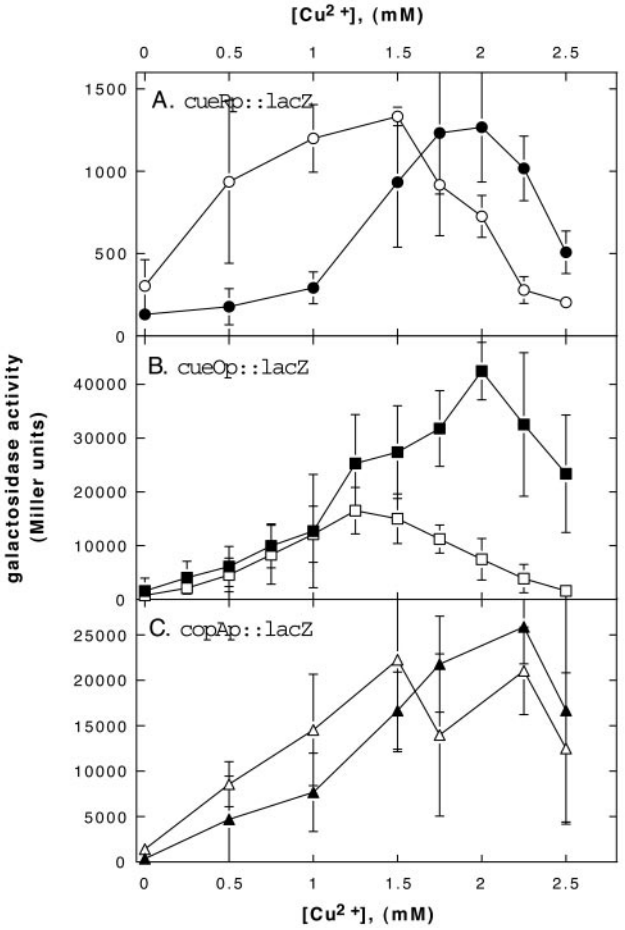

FIG. 3. Induction of copper homeostasis genes by metal cations in cells with and without RpoE. *E. coli* cells containing a *cueRp*::*lacZ* (panel A and circles), a *cueOp*::*lacZ* (panel B and squares) or a *copAp*::*lacZ* fusion (panel C and triangles) were treated with various concentration of Cu(II) in TGC medium, and the increase in β-galactosidase activity was recorded as Miller units. Cells contained the gene for the sigma factor RpoE (solid symbols) or not (open symbols). The mean values with standard deviation bars of three independent experiments performed in Tris-buffered mineral salts medium are shown.

lead resistance was unchanged in the Δ*rpoE* mutant strain (data not shown).

RpoE was induced by metals. A *rpoEp*::*lacZ* fusion was inserted as a single gene copy into the λ_{att} site of the chromosome of wild-type *E. coli* cells. When the resulting strain was treated with various metal cations in TGC medium, Cu(II) and Zn(II) resulted in a strong increase in reporter activity (data not shown). The copper-mediated increase in reporter gene activity was sigmoidal, with the strongest increase at the copper threshold concentration of about 1.5 mM Cu(II). The maximum induction coefficient reached was 26.3- ± 14.5-fold at 2.5 mM Cu(II). Zinc showed the strongest effect, with a maximum induction coefficient of 42.5- ± 16.6-fold at 0.8 mM Zn(II) and a sharp increase in reporter activity above 0.4 mM Zn(II). In contrast, reporter expression increased only slightly and as a linear function with Cd(II), Ni(II), Mg(II), or Na(I), and the last was used to induce osmotic stress (data not shown). The maximum induction coefficients obtained with these metals were 3.86- ± 1.91-fold at 100 μM Cd(II), 2.13- ± 0.34-fold at 1 mM Ni(II), 2.02- ± 0.69-fold at 50 mM Mg(II), and 4.52- ± 2.40-fold at 500 mM Na(I).

Global gene expression analysis. To analyze the full impact of the Δ*rpoE* deletion on metal homeostasis in *E. coli*, a global cellular expression analysis was performed with microarrays. Cells of the *E. coli* wild-type strain and its Δ*rpoE* deletion strain were treated in TGC medium for 10 min with 25 μM Cd(II), 100 μM Zn(II), 250 μM Cu(II), or without added metals prior to RNA isolation. This time should provide the cells sufficient opportunity to induce metal tolerance systems. The concentrations used were 10% of the maximally tolerated concentrations of the Δ*rpoE* mutant strain (Tables 1 and 3). In each microarray, 4,239 gene-specific spots were analyzed. Each condition was performed four times, which includes two independent cultivations of the *E. coli* strains and a dye swap experiment. The mean values of the four normalized expression values were then analyzed from different perspectives.

The mean expression values were evaluated in 10 comparisons focusing on (i) the comparison of Δ*rpoE* mutant and wild-type cells without added metals as a "baseline" (0±*rpoE*), (ii) the influence of copper on wild-type (WT±Cu) and mutant cells (Δ*rpoE*±Cu) considered separately before the global gene expression profile of copper-treated cells plus and minus RpoE was directly compared (Cu±*rpoE*), and (iii) the same procedure for cadmium (WT±Cd and Δ*rpoE*±Cd followed by Cd±*rpoE*) and (iv) zinc (WT±Zn, Δ*rpoE*±Zn, Zn±*rpoE*). In each comparison, a change in expression of more than 2.0 in either direction ($Q \geq 2.0$ or $Q \leq 0.5$) was counted as a significant change. Moreover, a significant change required that the difference between the two signals had to be larger than the sum of both signal deviations. Of the 4,239 genes analyzed, a quarter (1,069 genes) showed significant upregulation or downregulation with in at least one of the 10 comparisons (data not shown).

Changes in transcript levels in selected genes were validated with quantitative real-time qRT-PCR (Table 4). Due to the shorter incubation times and the lower metal concentrations used in the qRT-PCR experiments compared to the reporter gene experiments, only zinc was able to upregulate *rpoE* transcription (Table 4), while the *rpoEp*::*lacZ* reporter fusion was induced by all three metals. Compared to the microarray analy-

TABLE 3. Resistance of *E. coli* mutant strains to cadmium and zinc[a]

Relevant genotype	Zn^{2+}		Cd^{2+}	
	MIC (μM)	IC_{50} (μM)	MIC (μM)	IC_{50} (μM)
Wild type	1,400	859 ± 95 (85 ± 9)	900	572 ± 80 (109 ± 15)
Δ*rpoE*	1,100	616 ± 64 (136 ± 14)	300	248 ± 7 (91 ± 3)
Δ*zntA*	600	366 ± 21 (94 ± 5)	40	12 ± 4 (6 ± 2)
Δ*zitB*	1,400	899 ± 129 (94 ± 13)	900	546 ± 68 (123 ± 15)
Δ*rpoE* Δ*zntA*	600	206 ± 24 (43 ± 5)	40	6 ± 0 (1.3 ± 0.1)
Δ*zntA* Δ*zitB*	600	201 ± 11 (76 ± 4)	40	16 ± 3 (8.2 ± 1.7)
Δ*rpoE* Δ*zitB*	1,100	545 ± 107 (83 ± 16)	300	78 ± 26 (86 ± 29)
Δ*rpoE* Δ*zntA* Δ*zitB*	400	118 ± 1 (20 ± 0)	40	11 ± 1 (8.6 ± 1.0)

[a] MICs were determined on LB medium after 16 h at 37°C. The IC_{50} is the zinc or cadmium concentration required to decrease the turbidity at 600 nm of the growing cells to half of that of the positive control value (no added zinc), as shown for copper in Fig. 1. The number in parentheses following the IC_{50} value is slope *b* of the sigmoidal dose-response curve.

TABLE 4. Verification of microarray data by quantitative real-time RT-PCR analysis

Gene	Comparison	*Q* value		
		qRT-PCR[a]	Microarray I[b]	Microarray II[c]
rpoE	WT ± Zn	2.91 ± 1.02	4.52	2.98
	WT ± Cd	0.87 ± 0.23	<1.5	0.63
	WT ± Cu	1.23 ± 0.24	<1.5	1.03
yjal (zraP)	WT ± Zn	2,796 ± 630	327	228
	Δ*rpoE* ± Zn	1,774 ± 444	176	145
ompX	Zn ± *rpoE*	6.08 ± 2.38	3.04	3.20
b0805	Zn ± *rpoE*	0.42 ± 0.20	0.48	0.48
ompA	Cd ± *rpoE*	0.67 ± 0.15	0.35	0.38
	Cu ± *rpoE*	0.61 ± 0.21	0.50	0.53
	WT ± Zn	0.42 ± 0.17	0.47	0.82
rseA	Zn ± *rpoE*	0.12 ± 0.07	0.16	0.17
	Cu ± *rpoE*	0.48 ± 0.25	0.41	0.42
ompF	WT ± Zn	0.19 ± 0.13	0.10	0.07
	Δ*rpoE* ± Zn	0.17 ± 0.04	0.17	0.14
	Δ*rpoE* ± Cu	0.34 ± 0.07	0.46	0.39
ompC	0 ± *rpoE*	0.0069 ± 0.0020	0.04	0.035
	Zn ± *rpoE*	0.0070 ± 0.0014	0.06	0.065
	Cd ± *rpoE*	0.0039 ± 0.0006	0.04	0.043
	Cu ± *rpoE*	0.0031 ± 0.0007	0.03	0.03

[a] The results of four independent experiments, each performed in duplicate, are shown with standard deviations.
[b] As shown in Tables 5, 6, and 7 and in the supplementary material.
[c] Results of a microarray analysis with commercial software GeneSight 3.0 for comparison.

sis, qRT-PCR provided consistent data. One exception, *ompA*, was 2.9-fold downregulated ($Q = 0.35$, Table 4) in the Δ*rpoE* mutant strain upon cadmium treatment in the microarray approach. However, by generalized standards (a gene is defined as regulated when it shows more than a twofold change) it was not significantly regulated ($Q = 0.67$, Table 4) as determined by qRT-PCR. For all other genes tested, the qRT-PCR data confirmed differences in mutant and wild-type strain expression. The biases of regulation were similar, but the magnitude of regulation (up to 10-fold between microarray and qRT-PCR) varied notably. Differences in expression between microarray and qRT-PCR data were greater when regulation was stronger, with qRT-PCR usually giving the larger change in regulation. This observation has been reported by others (10, 20, 39, 42). As an independent technique, qRT-PCR generates more accurate and sensitive data and gives a wider dynamic range. No false positives could be detected among the genes tested. This and the narrow threshold of variability of the analysis marked the microarray data presented here as reliable.

Copper. When copper-treated cells of the Δ*rpoE* mutant cells were compared to copper-treated wild-type cells, mRNAs of only four genes appeared in lower quantities in the mutant cells (Table 5). The decreased concentration of *rpoE*-specific mRNA was a direct (and trivial) result of the method of *rpoE* deletion. Since RpoE controls expression of the *rpoErseABC* operon (2), the transcript level of *rseA* (encoding the RpoE-specific anti-sigma factor) was also affected. The gene with the strongest (33-fold, $Q = 0.03$, Table 5) decrease in mRNA concentration was the *ompC* gene, which encodes an outer membrane porin protein. Another gene encoding an outer membrane protein, *ompA*, was also downregulated, but only twofold. Validation with qRT-PCR showed strongly decreased *ompC* mRNA levels (140- to 320-fold) in untreated and metal-

TABLE 5. Changes in transcript levels in the Δ*rpoE* mutant strain and its wild-type strain after treatment with 250 μM Cu(II)[a]

Change	Gene	Description	Comparison (*Q* value)			
			+Cu/−Cu		Δ*rpoE*/WT	
			WT	Δ*rpoE*	−Cu	+Cu
Down regulation in Cu ± *rpoE* comparison	*ompC*	Outer membrane protein 1b (Ib; c)	1.56	1.28	0.04	**0.03**
	rpoE	Sigma-E factor; heat shock and oxidative stress	1.31	1.30	0.05	**0.05**
	rseA	Sigma-E factor, negative regulatory protein	1.56	0.98	0.66	**0.41**
	ompA	Outer membrane protein 3a (II*;G;d)	1.28	1.54	0.42	**0.50**
Up regulation in Cu ± *rpoE* comparison	*pspA*	Phage shock protein, inner membrane protein	1.10	1.15	2.75	**2.89**
	pspB	Phage shock protein	1.67	1.66	2.82	**2.80**
	sohA	Putative protease; htrA suppressor protein	0.88	1.02	1.84	**2.14**
	yjbE	ORF, hypothetical protein	1.04	1.04	2.88	**2.88**
	pspC	Phage shock protein: activates *psp* expression	1.47	1.31	2.48	**2.20**
	pspD	Phage shock protein	1.06	0.99	2.20	**2.04**
Upregulation in WT upon copper stress (WT ± Cu)[b]	*ylcC*	*cusF*, periplasmic copper-binding protein	**396.85**	350.18	0.94	0.83
	ylcB	*cusC*, OMF of the Cus system	**102.98**	54.55	1.35	0.72
	ybdE	*cusA*, RND of the Cus system	**15.30**	11.90	1.07	0.83
	ylcA	*cusR*, response regulator of *cus* 2,3-dihydro-2,3-dihydroxybenzoate	**10.59**	8.39	1.01	0.80
	entA	Dehydrogenase, enterobactin biosynthesis	**7.60**	5.62	1.13	0.83
	ybdB	ORF, hypothetical protein	**6.37**	4.98	1.07	0.83
	soxS	Regulation of superoxide response regulon	**6.24**	5.62	0.93	0.84
	fepA	Outer membrane receptor for ferric enterobactin and colicins B and D	**5.51**	3.62	1.19	0.78
	ylcD	*cusB*, MFP of the Cus system	**5.47**	4.42	0.96	0.78
Down regulation in WT upon copper stress (WT ± Cu)[c]	*yiaO*	Putative solute-binding transport protein	**0.14**	0.14	0.94	0.93
	b2875	Putative synthase	**0.15**	0.14	0.86	0.83
	ydeD	ORF, hypothetical protein	**0.15**	0.15	0.94	0.92

[a] The genes are listed by increasing or decreasing quotients of their message concentrations (*Q* values) in the Cu ± *rpoE* and WT ± Cu comparisons. All comparisons result from one data set. The *Q* values used for the ranking are identified by boldfacing. The full data set is available in the supplementary material.
[b] Nine top-ranking genes (of 61) are shown.
[c] Three top-ranking genes (of 229) are shown.

treated Δ*rpoE* mutant cells, but *ompA* transcript levels were also decreased only by half under the conditions tested (Table 4). A Δ*ompC* deletion strain displayed the same degree of copper sensitivity as a Δ*rpoE* single and also a Δ*ompC* Δ*rpoE* double deletion strain (Fig. 1A). Therefore, RpoE apparently contributes to copper resistance via increased expression of the outer membrane protein OmpC.

The contribution of OmpC to copper resistance is puzzling, because a porin is supposed to allow transport of substances across the outer membrane to the periplasm. Loss of OmpF and OmpC or of OmpF only (but not of OmpC alone) led to increased silver resistance in *E. coli* (36), which was based on diminished accumulation of Ag(I). If OmpC is able to transport Cu(II), the absence of OmpC should lead to decreased copper uptake and thus to increased resistance to this cation rather than increased sensitivity. Spontaneous copper-resistant *E. coli* mutants did not contain "outer membrane protein b" (37). However, OmpC and another major *E. coli* porin, OmpF, run close together on sodium dodecyl sulfate gels (36), so lack of "protein b" in this publication may indicate a loss of OmpC and of OmpF in these copper-resistant mutants (compare Fig. 1 in reference 37) with the corresponding figure in reference 36). One explanation of this complicated situation could be that *E. coli* is able to control copper transport by OmpC to some extent but not by other porins.

Six genes were upregulated in the Cu±*rpoE* comparison (Table 5). Four genes encode the components (PspA, PspB, PspC, and PspD) of the phage shock response system Psp. The phage shock system Psp is strongly expressed in response to stressful environmental conditions, such as heat shock, ethanol treatment, osmotic shock, filamentous phage infection (64), and secretion defects (30). While PspB and PspC are involved in regulation of the Psp system, PspA protects the cell against dissipation of the proton motive force under stress conditions, probably by maintaining the integrity of the inner membrane (1, 33).

The expression change of only one gene, *sohA*, was specific for copper. This gene encodes a putative protease and an *htrA* suppressor. It allows cold-sensitive *htrA* mutant cells to grow at 42°C (6). However, the increase in expression of *sohA* in the Cu±*rpoE* comparison was just above the twofold cutoff level.

Thus, only 10 genes were changed in expression when copper-treated *rpoE* mutant and wild-type cells were compared. All 10 showed a similar change mRNA concentration in the 0±*rpoE* comparison (*sohA*, but only weakly, with $Q = 1.8$). Therefore, the altered mRNA concentrations of these genes were an effect of the Δ*rpoE* deletion and not of the copper treatment.

Copper treatment of wild-type cells resulted in upregulation of 61 genes (see the supplemental material). The *cus* copper resistance operon genes and genes involved in enterobactin synthesis and iron uptake showed the strongest upregulation (Table 5). In the presence of copper, CueO protects *E. coli* cells by oxidizing enterobactin, the catechol iron siderophore of *E. coli* (26), which the cell might counteract by increased enterobactin synthesis under copper stress. Cus mediates transenvelope efflux of copper by the CusCBA protein complex (21, 22, 25, 51). CusR is the response regulator of the determinant (21, 44), and its histidine sensor kinase CusS (YbcZ) was induced 1.7-fold by copper. CusF (YlcC) showed the strongest response upon copper shock and is a periplasmic copper-binding protein and maybe a periplasmic copper chaperone (22). Vigorous induction of the *cus* determinant by copper matches data obtained elsewhere (21, 22, 51).

Induction of the *cus* determinant by copper was accompanied by upregulation of other genes putatively involved in copper homeostasis. The *yedWV* genes encoding the putative copper response two-component regulatory system CopRS were also induced two- to threefold after copper treatment. The gene for the periplasmic oxidase CueO (YacK) was induced 1.6-fold, and that of the copper-exporting P-type ATPase CopA (YbaR) was induced 5-fold. In contrast, the genes that were downregulated in copper-treated wild-type cells (total number, 229 genes) did not show any evident connection to copper homeostasis (supplemental material and Table 5).

All genes displaying a change in expression upon copper treatment in wild-type cells also showed a similar change in expression in the Δ*rpoE* mutant cells, but the change of gene expression in the mutant cells seemed to be smaller than that in the wild-type cells (Table 4). To analyze this in more detail for all *E. coli* genes, the change in gene expression upon copper treatment of Δ*rpoE* mutant cells in the Δ*rpoE*±Cu comparison (Q_{CuMut}) was plotted against the corresponding value in the WT±Cu comparison (Q_{CuWT}) in a double $\log_{10}$ plot (Fig. 4A in the supplemental material). Most of the gene values clustered around $\log_{10} = 0$, meaning no change in expression has occurred in either strain. Linear regression of these more than 4,000 data points resulted in a slope of 0.845. This means that the change in gene expression into both directions is smaller in Δ*rpoE* mutant cells than in the wild type, with $Q_{\mathrm{CuMut}} = Q_{\mathrm{CuWT}}{}^{0.845}$. Similar results were obtained for the data of the cadmium- and zinc-specific comparisons (Fig. 4B and C in the supplemental material), which indicates a similar effect of the Δ*rpoE* deletion on resistance to all three metals tested.

Thus, *E. coli* responded to copper stress primarily with synthesis of the Cus transenvelope efflux complex, the CusF periplasmic copper binding protein, and some genes involved in iron metabolism. Deletion of *rpoE* led to decreased flexibility in the global transcriptional adaptation to copper stress and to copper-mediated killing of *E. coli*, which affected the lag phase of growth. Second, it led to decreased expression of *ompC* in copper-treated as well as untreated cells, which also resulted in decreased copper resistance, maybe by enhanced copper transport by other porins, as discussed in detail above.

Cadmium. When the global transcription profile of cadmium-treated Δ*rpoE* mutant cells was compared to that of cadmium-treated wild-type cells, decreased expression of eight genes was found (Table 6), including *ompC*, *rpoE*, and *ompA*, which also appeared in the Cu±*rpoE* comparison and the 0±*rpoE* comparison and were discussed above. As in the case of copper, deletion of *ompC* led to decreased cadmium resistance, and the diminished resistance of the Δ*ompC*, Δ*rpoE*, and Δ*ompC*Δ*rpoE* mutant strains was similar (Fig. 1B).

The genes *katG*, *grxA*, and *sbp* showed a unique regulatory pattern, because they were upregulated in cadmium-treated wild-type cells but only upregulated by cadmium to a significantly smaller extent in the Δ*rpoE* mutant cells. Five genes were upregulated in the Cd±*rpoE* comparison; four of them

TABLE 6. Changes in transcript levels in the Δ*rpoE* mutant strain and its wild-type strain after treatment with 25 μM Cd(II)[a]

Change	Gene	Description	Comparison (*Q* values)			
			+Cd/−Cd		Δ*rpoE*/WT	
			WT	Δ*rpoE*	−Cd	+Cd
Down regulation in Cd ± *rpoE* comparison[b]	*ompC*	Outer membrane protein 1b (Ib;c)	0.90	0.96	0.04	**0.04**
	rpoE	RNA polymerase, sigma-E factor	0.76	0.75	0.05	**0.05**
	ompA	Outer membrane protein 3a (II*;G;d)	1.68	1.41	0.42	**0.35**
	katG	Catalase; hydroperoxidase HPI(I)	4.19	1.56	1.02	**0.38**
	grxA	Glutaredoxin 1	4.90	2.30	0.86	**0.40**
Upregulation in Cd ± *rpoE* comparison	*yjbE*	ORF, hypothetical protein	0.79	1.13	2.88	**4.13**
	pspA	phage shock protein, inner membrane protein	1.07	1.30	2.75	**3.33**
	pspB	phage shock protein	1.68	1.79	2.82	**3.01**
	pspC	phage shock protein: activates phage shock protein expression	1.56	1.54	2.48	**2.44**
	b1481	ORF, hypothetical protein	1.38	1.68	1.92	**2.33**
Upregulation in WT upon cadmium stress (WT ± Cd)[c]	*ycfR*	ORF, hypothetical protein	**25.19**	5.97	1.89	0.45
	b1973	*zinT*(*yodA*), zinc binding outer membrane protein	**21.87**	23.41	1.06	1.14
	yebL	*znuA*	**12.73**	13.74	0.93	1.01
	Ftn	*ftnA*, cytoplasmic ferritin (iron storage protein)	**11.84**	9.13	0.98	0.75
	zntA	Zinc-transporting ATPase	**7.66**	8.58	0.99	1.11
	cysH	3′-Phosphoadenosine 5′-phosphosulfate reductase	**5.07**	4.03	0.78	0.62
Downregulation in WT upon cadmium stress (WT ± Cd)[d]	*yhdV*	ORF, hypothetical protein	**0.11**	0.12	0.95	0.97
	ydeD	ORF, hypothetical protein	**0.12**	0.13	0.94	0.99
	fliP	Flagellar biosynthesis	**0.12**	0.13	0.90	0.95

[a] The genes are listed by increasing or decreasing quotients of their message concentrations (*Q* values) in the Cd ± *rpoE* and WT ± Cd comparisons. All comparisons result from one data set. The Q values used for the ranking are identified by boldfacing. The full data set is available in the supplementary material.
[b] Top-ranked five of eight genes.
[c] Top-ranked 6 of 133 genes.
[d] Top-ranked 3 of 302 genes.

(*yjbE* and three *psp* genes) were similarly upregulated in the Cu±*rpoE* comparison.

A total of 133 genes were upregulated in cadmium-treated wild-type cells (supplemental material). The *ycfR* gene showed the strongest upregulation. YcfR might be an outer membrane protein with an unknown function. Open reading frame *b1973* in Table 6 is *yodA* and encodes a zinc/cadmium-binding, lipocalin-like protein (17). This gene is also known as *zinT*, encoding a zinc-binding protein, which likely functions as an alternative or additional zinc-chelating component of the zinc ZnuABC transporter (53). These two genes are followed by *yebL*, encoding ZnuA, the periplasmic binding protein of the ZnuABC zinc uptake system, and *ftnA*, which encodes the iron storage protein ferritin. The next gene is *zntA*, encoding the zinc- and cadmium-exporting P-type ATPase ZntA (56), which is involved in cadmium resistance. These data may indicate an action of cadmium on cellular zinc and iron metabolism, since *E. coli* responded to cadmium stress with increased expression of zinc uptake and cadmium efflux systems. Cadmium also induced expression of ZinT (YodA), ZnuA, Ftn, and ZntA in the Δ*rpoE* mutant strain (Table 6).

In *E. coli* wild-type cells, cadmium also induced genes required for cysteine biosynthesis and redox metabolism (*grxA* and *katG*). A total of 302 genes were downregulated in wild-type cells after cadmium treatment (Table 6 and supplemental material). Most of the genes with a change in expression in wild-type cells were also differentially expressed in Δ*rpoE* mutant cells as a response to cadmium (supplemental material).

Thus, *E. coli* responded to cadmium stress with increased synthesis of the cadmium efflux pump ZntA, increased zinc and iron binding and uptake, and cysteine, glutaredoxin 1, and hydroperoxidase I production. Deletion of *rpoE* diminished adaptation specifically to cadmium by preventing increased cysteine, glutaredoxin 1, and hydroperoxidase I synthesis. The flexibility of general stress adaptation and *ompC* mRNA levels were also decreased as in the case of copper stress.

Zinc. In the direct comparison of zinc-treated cells with and without *rpoE*, expression of 17 genes was down- and that of 16 genes was upregulated (Table 7). Expression of 17 of these 33 genes was also changed in the 0±*rpoE* comparison. Therefore, their change in expression was a result of the *rpoE* deletion rather than of zinc treatment. This was also true for the first two members of the list of downregulated genes, *rpoE* and *ompC*. In contrast to the situation with copper and cadmium, a Δ*rpoE* Δ*ompC* double-deletion strain was more sensitive to zinc than either single-deletion strain (Fig. 1C). This suggests two independent pathways of OmpC- and RpoE-mediated resistance to zinc.

In the list of downregulated genes in the Zn±*rpoE* comparison, *rpoE* and *ompC* were followed by *htrA* (Table 7 and supplemental material). Expression of the heat shock serine protease HtrA (= DegP) is under RpoE control and required under various stress conditions such as hyperosmotic shock, virulence, oxidative stress, and dead cell lysis (7, 29, 49, 61, 62). HtrA is essential for *E. coli* at elevated temperatures and is also involved in regulation of activity of RpoE because it destabilizes the RpoE regulatory component RseA (4, 35). Proteins related to HtrA are widely distributed, from bacteria to plants (13, 31), and the crystal structure indicates that a temperature-dependent conformational change might be required to activate the protease function of this protein (32, 34). The *htrA* gene was induced by Zn(II) 11-fold in the wild type, but

TABLE 7. Changes in transcript levels in the Δ*rpoE* mutant strain and its wild-type strain after treatment with 100 μM Zn(II)[a]

Change	Gene	Description	Comparison (*Q* values)			
			+Zn/−Zn		Δ*rpoE*/WT	
			WT	Δ*rpoE*	−Zn	+Zn
Down regulation in Zn ± *rpoE* comparison[b]	*rpoE*	RNA polymerase, sigma-E factor	4.52	1.88	0.05	**0.02**
	ompC	Outer membrane protein 1b (Ib;c)	1.05	1.59	0.04	**0.06**
	htrA	Periplasmic serine protease HtrA = DegP	11.41	2.20	0.83	**0.16**
	rseA	Sigma-E factor, negative regulatory protein	5.08	1.23	0.66	**0.16**
	ychH	ORF, hypothetical protein	5.02	0.36	3.63	**0.26**
Upregulation in Zn ± *rpoE* comparison[c]	*pspA*	Phage shock protein, inner membrane protein	1.76	2.11	2.75	**3.29**
	ompX	Outer membrane protein X	0.57	1.32	1.31	**3.04**
	ycfS	ORF, hypothetical protein	1.39	2.96	1.40	**2.98**
	pspB	Phage shock protein	2.81	2.95	2.82	**2.96**
	pspC	Phage shock protein: activating function	1.89	2.16	2.48	**2.83**
Upregulation in WT upon zinc stress (WT ± Zn)[d]	*yjaI*	*zraP*	**326.78**	75.94	1.18	0.63
	htrA	Periplasmic serine protease HtrA	**11.41**	2.20	0.83	0.16
	entA	2,3-Dihydro-2,3-dihydroxybenzoate dehydrogenase, enterobactin biosynthesis	**10.12**	4.96	1.13	0.55
	ybdB	ORF, hypothetical protein	**7.02**	3.80	1.07	0.58
	entB	2,3-Dihydro-2,3-dihydroxybenzoate synthetase, enterobactin biosynthesis	**6.67**	2.92	1.27	0.55
	nrdE	Ribonucleoside-diphosphate reductase 2, alpha subunit, enterobactin biosynthesis	**5.53**	3.64	1.04	0.69
	fhuE	Outer membrane receptor for ferric siderophore uptake	**5.48**	3.24	1.24	0.73
Down regulation in WT upon zinc stress (WT ± Zn)[e]	*ompF*	Outer membrane protein 1a (Ia;b;F)	**0.10**	0.17	0.62	1.05
	rpsU	30S ribosomal subunit protein S21	**0.19**	0.79	0.38	1.58
	ymbA	ORF hypothetical protein	**0.20**	0.27	0.70	0.92

[a] The genes are listed by increasing or decreasing quotients of their message concentrations (Q values) in the Zn ± *rpoE* and WT ± Zn comparisons. All comparisons result from one data set. The Q values used for the ranking are identified by boldfacing. The full data set is available in the supplementary material.
[b] Top-ranked 5 of 17 genes.
[c] Top-ranked 5 of 16 genes.
[d] Top-ranked 8 of 256 genes.
[e] Top-ranked 6 of 115 genes.

expression was decreased 6-fold when zinc-treated mutant and wild-type cells were compared (Table 7, $Q = 0.16$). This may indicate that *htrA* transcription is regulated by both RpoE and Zn(II).

The expression of the HtrA target, the RpoE regulator RseA, was also zinc dependent. The transcript level of *rseA* was not changed in the 0±*rpoE* comparison, fivefold increased in the WT±Zn comparison, but not increased at all under the same conditions in the mutant (Table 7). Consequently, this gene appeared to be sixfold downregulated ($Q = 0.16$) in the Zn±*rpoE* comparison. This was also verified by qRT-PCR (Table 4).

As in the Cu±*rpoE*, Cd±*rpoE*, and 0±*rpoE* comparisons, *psp* transcripts were increased in the Zn±*rpoE* comparison. The remaining Zn±*rpoE* upregulated genes have mostly unknown functions. OmpX (threefold upregulated as determined by microarrays, Table 5, and sixfold by qRT-PCR, Table 4) was named because of its similarity to OmpX from *Enterobacter cloacae* (63). Thus, in addition to OmpC, other outer membrane proteins might also be involved in metal homeostasis.

In wild-type cells, 256 genes were upregulated upon addition of zinc (Table 7 and supplemental material). The highest increase was an exceptional 327-fold for *yjaI (zraP)*, which encodes a zinc-dependent periplasmic protein (50). The *yjaI (zraP)* gene was also induced by zinc in the Δ*rpoE* mutant strain (176-fold). A strong increase in the *zraP* mRNA under this condition in wild-type and mutant cells was verified by qRT-PCR (Table 4).

Genes involved in ferrous iron transport were upregulated by zinc treatment in wild-type cells: *feoA*, *entA*, *entB*, *entE*, and *entF*, involved in ferrous iron uptake and biosynthesis of the catecholate siderophore enterobactin, and the genes for outer the membrane receptors, *fhuE* and *fepA*, involved in iron-siderophore uptake, were all upregulated (Table 7). The same genes were also upregulated in Δ*rpoE* cells, but to a smaller degree. All the data together may indicate that zinc treatment results in iron depletion and that the *rpoE* mutant can only partially adjust to this stress condition.

The *zntA* gene was induced only twofold in wild-type cells (supplemental material), and expression of the *zitB (ygbR)* gene, encoding the second, proton-driven Zn(II) efflux system of *E. coli* (23), was not changed under these conditions. This matched the result of the mutant studies (Table 3), which indicated that increased ZitB expression may not be required for zinc resistance when RpoE and ZntA were present.

The 115 genes downregulated in wild-type cells upon addition of zinc (Table 7) were led by *ompF* (10-fold downregulation, $Q = 0.1$), which was also number one in the corresponding list of the Δ*rpoE* strain (Table 7). Its downregulated expression after zinc treatment of both types of cells was also verified by qRT-PCR (Table 4). The expression level of both genes, *ompC* and *ompF*, is determined by a two-component regulatory system composed of the membrane-bound histidine kinase EnvZ and the response regulator OmpR (11). The amount of *ompR*-specific mRNA increased twofold in Δ*rpoE* and wild-type cells (only by zinc, not by cadmium or copper), but that of *envZ* remained unchanged (supplemental material). Thus, concerning porins, the cellular response to zinc seems to be more complicated than that to copper. The decrease in the OmpC level as a consequence of the *rpoE* deletion was connected to a zinc-dependent downregulation of *ompF* in both

cell types. Additionally, *ompX* messages increased threefold in the Zn±*rpoE* comparison. These data show that *E. coli* may changed its porin composition as a response to metal stress.

Conclusion. Expression of RpoE was induced by metals and a Δ*rpoE* mutant strain showed decreased metal resistance. Two possible modes of involvement of RpoE in metal homeostasis were found, (i) flexibility of the cell to adapt the transcriptome to stress conditions (Fig. 4 and supplemental material) and (ii) production of outer membrane proteins. The first mode would explain the increased lag phase of growth of Δ*rpoE* mutant cells in the presence of copper (Fig. 2). From copper via cadmium to zinc, the situation becomes more and more complicated, with additional categories of genes being involved and changed in expression after *rpoE* deletion in different ways. However, decreased transcriptome flexibility and decreased expression of OmpC are two effects of Δ*rpoE* deletion on cellular copper homeostasis in *E. coli* that were clearly demonstrated.

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